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Inhibiting Systemic Autophagy during Interleukin 2 Immunotherapy Promotes Long-term Tumor Regression

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Abstract

Administration of high-dose interleukin-2 (HDIL-2) has durable antitumor effects in 5% to 10% of patients with melanoma and renal cell carcinoma. However, treatment is often limited by side effects, including reversible, multiorgan dysfunction characterized by a cytokine-induced systemic autophagic syndrome. Here, we hypothesized that the autophagy inhibitor chloroquine would enhance IL-2 immunotherapeutic efficacy and limit toxicity. In an advanced murine metastatic liver tumor model, IL-2 inhibited tumor growth in a dose-dependent fashion. These antitumor effects were significantly enhanced upon addition of chloroquine. The combination of IL-2 with chloroquine increased long-term survival, decreased toxicity associated with vascular leakage, and enhanced immune cell proliferation and infiltration in the liver and spleen. HDIL-2 alone increased serum levels of HMGB1, IFN- γ , IL-6, and IL-18 and also induced autophagy within the liver and translocation of HMGB1 from the nucleus to the cytosol in hepatocytes, effects that were inhibited by combined administration with chloroquine. In tumor cells, chloroquine increased autophagic vacuoles and LC3-II levels inhibited oxidative phosphorylation and ATP production and promoted apoptosis, which was associated with increased Annexin-V⁺/propidium iodide (PI)⁻ cells, cleaved PARP, cleaved caspase-3, and cytochrome *c* release from mitochondria. Taken together, our findings provide a novel clinical strategy to enhance the efficacy of HDIL-2 immunotherapy for patients with cancer. *Cancer Res*; 72(11); 2791–801. ©2012 AACR.

Introduction

Two decades ago, recombinant interleukin-2 (IL-2) received U.S. Food and Drug Administration approval for the treatment of patients with advanced renal cancer and subsequently of patients with melanoma. High-dose IL-2 (HDIL-2) administration is associated with an objective 25% response rate in patients with kidney cancer, as reported in the recently completed IL-2 SELECT trial (1). Almost 20% of these patients survive more than 5 years (2–4). Attempts to improve the response rate and/or limit toxicity of IL-2 administration by inhibiting TNF, iNOS, or VEGF have failed. Combination with IFN- α administration did not improve outcome appreciably (2, 3). Other efforts including vaccination (5), adoptive cellular therapies (6), and CTLA-4 inhibition (7, 8) are associated with both increased efficacy and toxicity. HDIL-2 administration

remains the only agent with proven efficacy in producing durable complete and partial responses in patients with metastatic renal cell carcinoma (RCC; ref. 9).

The greatest limitation of IL-2 treatment has been the associated side effects including hypotension as well as cardiac, gastrointestinal, renal, cerebral, pulmonary, and hepatic toxicity. These adverse effects are occasionally life threatening, and treatment is usually restricted to specialized centers, often resulting in early discontinuation or interruption of treatment (10, 11). The precise mechanism mediating these side effects has not been clear. We have proposed that IL-2 toxicity is due to a cytokine-induced systemic autophagic syndrome. Recently, several cytokines including type II IFN and TGF- β have been shown to induce autophagy (12). We hypothesized that the systemic syndrome associated with IL-2 treatment was related to cytokine-induced autophagy and temporally limited tissue dysfunction. The use of the autophagy inhibitor, chloroquine could limit toxicity and thereby enhance efficacy.

Autophagy is a tightly regulated catabolic process involving the degradation of cellular components through the lysosomal machinery and plays a central role in cell growth, development, and homeostasis (13). The role of autophagy in cancer is complex and context dependent. In normal tissues, autophagy is an important tumor suppressor pathway that limits oxidative stress and tissue damage that can promote cancer initiation during periods of excessive apoptotic cell death and inflammation. Autophagy, however, also supports cellular metabolism that has the potential to aid the growth of

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advanced tumors with increased metabolic demands following an "autophagic switch" (14) in developing tumors. In an established tumor, cells are exposed to perpetual hypoxia, acidosis, and nutrient deprivation. Increased autophagic flux enables adaptation to the hypoxic and nutrient-limited micro-environment. Increased autophagy is observed clinically in late-stage colon cancer, breast cancer, melanoma, hepatoma, and malignant glioma (15–19). Enhanced immune responses to tumors have been observed when hypoxia-induced autophagy is inhibited by chloroquine treatment (20). Autophagy promotes metastasis in some circumstances, enhancing tumor cell fitness in response to environmental stress (21).

Several studies have suggested that autophagy may act as a protective mechanism in tumor cells in which cell death is induced by chemotherapy, immunotherapy, or radiotherapy (20, 22–25). Targeting autophagy has increased the antitumor effects of individuals anticancer therapies in preclinical trials (13). There has been very limited exploration of autophagy inhibition therapy in combination with biologic therapy (20, 26).

Chloroquine was used for many years as an antimalarial but is now used most commonly in patients with rheumatoid arthritis and systemic lupus erythematosus (SLE). It inhibits autophagy by blocking acidification of the lysosome, preventing fusion with the autophagosome (23). This results in decreased degradation of autophagosomes, eventuating in either apoptotic or necrotic cell death. Chloroquine has extensive biologic effects, inhibiting cellular proliferation and/or inducing apoptosis in human and murine tumor cell lines (24, 27). Induction of apoptosis is associated with the loss of mitochondrial membrane potential, release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of PARP. *In vivo*, chloroquine significantly inhibits 4T1 colorectal cancer growth and metastasis in murine models and induces apoptosis within the tumor microenvironment (28). Studies of human (24, 29) and murine cancer cell lines (30) suggest that chloroquine may exert significant antitumor activity by inhibiting the induction of autophagy following cancer therapy. We hypothesized that inhibition of autophagy with chloroquine in combination with HDIL-2 treatment would increase antitumor effects and promote survival when compared with IL-2 administration alone, enabling more effective expansion and function of immune cells.

Materials and Methods

Animals and tumor cell lines

Female C57BL/6 (B6, H-2^b) mice, 8- to 10-week-old, were purchased from Taconic. Animals were maintained in a specific pathogen-free facility at the University of Pittsburgh Cancer Institute (Pittsburgh, PA) and used in accordance with institutional and NIH guidelines. MC38 murine colorectal carcinoma and Panc02 adenocarcinoma cells (C57BL/6 syngeneic) were purchased from The American Type Culture Collection. Renca renal cell cancer and B16 melanoma cell lines were gifts from Dr. W. Storkus at the University of Pittsburgh. All these cell lines were authenticated using genomic profiling in March 2012 (IDEXX Radil Cell Check). Cells

were maintained in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 5% heat-inactivated FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate.

Liver metastasis model

Liver metastases were obtained by direct portal injection of tumor cells as described previously (31). Briefly, mice were anesthetized with a single intraperitoneal injection of ketamine (50 mg/kg, NLS animal Health) and xylazine (10 mg/kg, NLS animal Health). The portal vein was exposed through a small midline incision. A total of 2×10^5 luciferase-transfected tumor cells suspended in 200 μ L normal saline were injected. The incision is closed with vicryl suture. Seven days following tumor inoculation, mice were randomized into 6 groups and received their first bioluminescence imaging (BLI) measurement, then started receiving intraperitoneal injection of rIL-2 with or without combination of chloroquine. Clinical grade rIL-2 was a kind gift of Prometheus Laboratories Inc. Untreated control mice (UT) were injected with a comparable amount of normal saline on the same schedule. Tumor burden was assessed with the IVIS bioluminescence image described later. Blood was collected by direct intracardiac puncture and spleens and livers were harvested for electron microscopy, confocal imaging, and isolation of immune cells.

Luciferase transfection of tumor cells and BLI

Stably transduced tumor cells expressing the firefly luciferase gene were generated by lentiviral transfection of the pGL4 Luciferase Reporter Vector (Promega) and selected with puromycin. Growth characteristics and phenotype of the transduced cells were compared with the parental strain *in vitro* to verify the absence of any effects secondary to retroviral insertion. Before imaging, mice were anesthetized by isoflurane (Wester Veterinary) inhalation followed by intraperitoneal injection of luciferin (300 mg/kg; Caliper Life Sciences). After waiting 8 minutes to allow proper distribution of luciferin, the mice were imaged with an IVIS 200 system (Xenogen Corporation) according to the manufacturer's instructions. Living Image software (Xenogen) was used to analyze the resultant data. Regions of interest were manually selected and quantification is reported as the average of photon flux within regions of interest. The BLI signal is represented as photons/s/cm²/Sr.

Isolation of nonparenchymal cells and flow cytometry

Mouse livers were minced and digested with 1% collagenase (Sigma) solution at 37°C for 30 minutes. To obtain adequate numbers of nonparenchymal cells, livers from 3 to 5 animals were combined from each treatment group. The nonparenchymal cells were then isolated by centrifugation over a Percoll gradient (Sigma Chemical Co.). Cell surface antigen expression was analyzed by flow cytometry (Becton Dickinson FACSscan) using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies against mouse CD11c, CD14, CD19, CD4, CD8, Gr-1, and NK1.1 (all from BD Pharmingen). Appropriate isotype and species-matched irrelevant monoclonal antibodies were used as controls.

Serum cytokine determination

Blood was collected from direct intracardiac puncture at individual intervals following tumor inoculation. Serum was used to measure HMGB1 (Shinotest), IL-6, IL-18, and IFN- γ (R&D) levels by ELISA.

Detection of apoptosis

MC38 tumor cells (2×10^5 /mL) were cultured in 24-well plates and treated with chloroquine for 4 or 24 hours. Cells were then harvested and stained with Annexin-V and propidium iodide (PI; BD Pharmingen) according to the manufacturer's protocol. Quantitative analysis was conducted by flow cytometry, with 10,000 events acquired from each sample.

Immunofluorescent staining

A portion of each lobe of the liver was embedded in OCT Compound (Miles), frozen, and stored at -80°C . Cryostat sections (8 μm) were used for immunofluorescent evaluation. *In vitro*, tumor cells were cultured in 8-chamber slides, treated with 100 to 200 $\mu\text{mol/L}$ chloroquine for 4 and 24 hours, fixed with 2% paraformaldehyde (PFA) for 30 minutes, and prepared for immunofluorescent staining. The following primary monoclonal antibodies were used: rabbit anti-human HMGB1 (R&D) and rabbit anti-LC3 (Novus Biologicals Inc.), rabbit-TOM-20 and mouse anti-cytochrome *c* (Santa Cruz Biotechnology Inc.). Slides were incubated with the primary antibody overnight at 4°C . Following 3 washes in PBS, slides were incubated with fluorescent-conjugated secondary antibodies for 45 minutes followed by Hoechst nuclear staining. Negative controls included staining with the corresponding isotype for each antibody and staining with secondary antibody alone. Positive controls included immunostaining of known positive tissues.

Protein blot analysis

Whole-cell lysates were resolved on 10% SDS-PAGE gel and transferred to 0.2 μm nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies specific for cleaved PARP (Cell Signaling), caspase-3, (Assay designs Inc.), LC-3 (Novus Biologicals), cytochrome *c* (Santa Cruz), and β -actin (Sigma). After incubation with peroxidase-conjugated secondary antibodies for 1 hour at 25°C , membranes were developed with the SuperSignal West Pico chemiluminescence kit (Pierce) and exposed to film. ImageJ was used to quantify the bands.

Statistical analyses

Statistical significance was assessed using the Student *t* test, Mann-Whitney *U* test, or ANOVA when appropriate with SPSS 16.0 (SPSS) or Spotfire DecisionSite (Tibco). A *P* value less than 0.05 was considered significant. All experiments reported here were repeated at least 2 or 3 times with similar results with representative findings presented.

Transmission electron microscopy (TEM; refs. 32, 33), ATP quantification, and XF Bioenergetic Assay were used and are described in Supplementary Materials.

Results

Chloroquine, in combination with HDIL-2, promotes profound antitumor effects, enhancing murine survival in a liver metastasis model

In preliminary experiments, we confirmed that rIL-2 inhibited tumor growth in a dose-dependent fashion (Supplementary Fig. S1) in a murine liver metastasis tumor model that we have developed. Although administration of 600,000 IU per mouse rIL-2 twice a day can inhibit tumor growth, many of these mice subsequently progress (Fig. 1A and B). Administration of high-dose rIL-2 resulted in life-threatening systemic toxicity, which precluded administration of higher doses. We hypothesized that these adverse effects may be related to the widespread induction of systemic autophagy. Therefore, we sought to determine the effects of administration of autophagy inhibitor agent chloroquine both alone and in combination with IL-2, in a hepatic metastatic tumor model.

Mice received 2×10^5 luciferase-labeled mouse colorectal cancer MC38 cells via portal vein injection. Seven days later, they were randomly divided into 6 groups that received vehicle control (UT), chloroquine alone 50mg/kg/d for 30 days, rIL-2 60,000 (low-dose IL-2, LDIL-2) or 600,000 IU per mouse (HDIL-2), twice a day for 5 day, with or without combination of chloroquine. Tumor growth was measured by BLI (Fig. 1A and B) and survival of mice was determined (Fig. 1D and Table 1). We found that 50 mg/kg chloroquine alone only had a modest but insignificant effect in inhibiting tumor growth ($P = 0.44$). In the UT control group, median survival was 31 days and the longest survival time was 55 days. IL-2 administration inhibited tumor growth in a dose-dependent manner. Low-dose IL-2 only modestly inhibited, whereas HDIL-2 significantly inhibited, tumor growth, prolonging the resultant survival time ($P < 0.01$). The median survival in the HDIL-2 group was 135 days and 44.4% of animals were tumor free, surviving longer than 150 days.

A dramatic effect on tumor growth was noted when chloroquine was administered in combination with HDIL-2. Although BLI revealed visible tumors in all 5 mice on day 7 before treatment in the liver (Fig. 1A and B), after 5 days of HDIL-2 and chloroquine combination treatment, only one mouse developed tumor whereas the others were completely eradicated (90% of animals) and survived without tumor for more than 150 days (Table 1). Comparing the survival curves in these 2 groups reveals a significant difference ($P = 0.024$). These results suggest that the combination strategy of HDIL-2 and chloroquine was extraordinarily effective with almost complete elimination of tumors (Fig. 1A). Similar antitumor effects of IL-2 were observed with treatment of the pancreatic cancer cell line Panc02 in the hepatic tumor model (Supplementary Fig. S2) as well as the Renca tumor in BALB/c mice (Supplementary Fig. S3). The B16 melanoma pulmonary metastases model was not susceptible to IL-2 alone (as shown previously by us and others) or in combination with chloroquine (data not shown).

In the clinic, one of the primary adverse effects of HDIL-2 therapy is the vascular leak syndrome resulting in fluid retention associated with increased body weight. To assess HDIL-2 toxicity, murine body weight was measured before and every

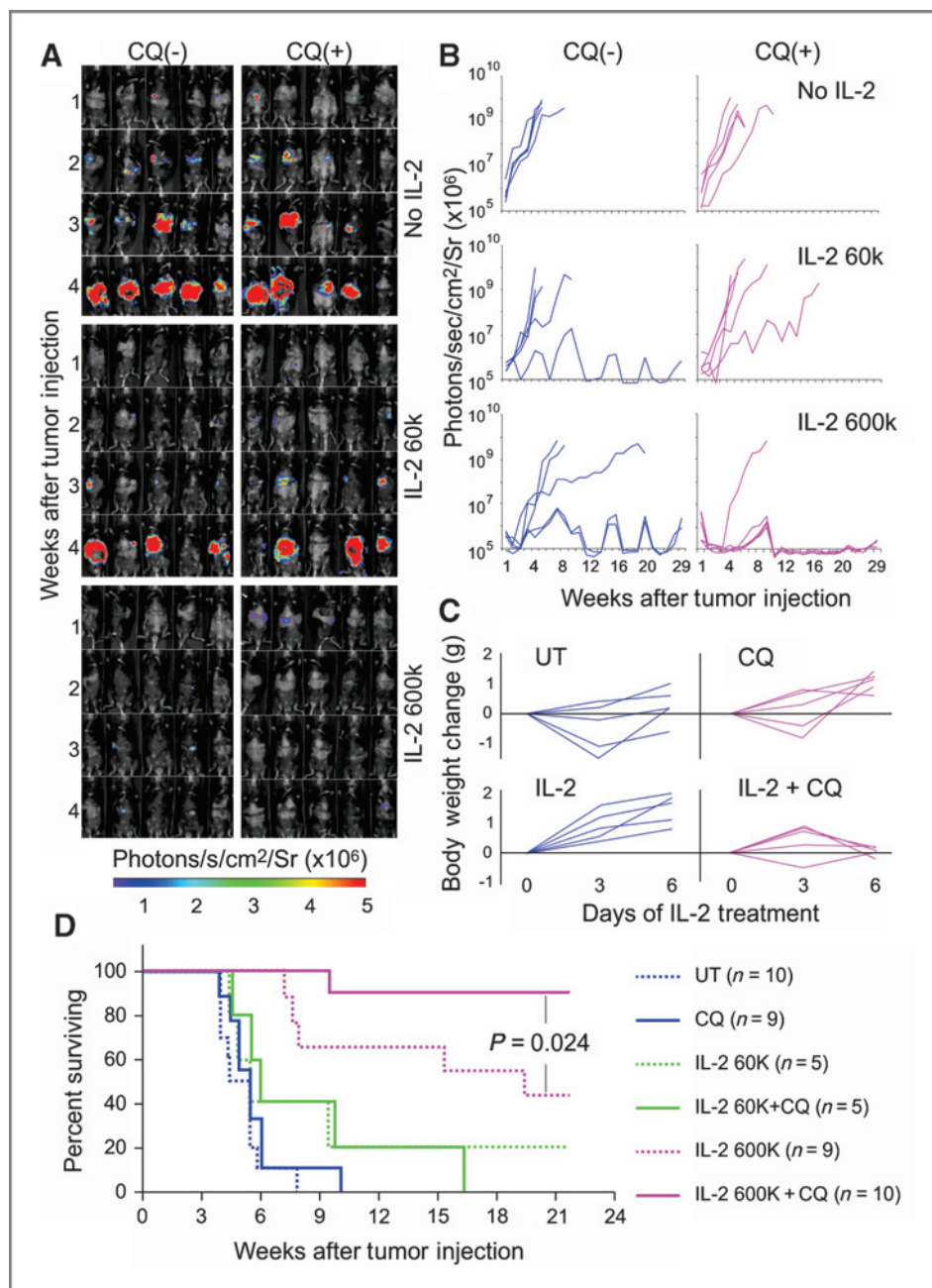


Figure 1. Chloroquine combination with rIL-2 markedly enhances antitumor effects, prolonging survival time in a murine hepatic metastasis model. C57BL/6 mice received 2×10^5 per mouse luciferase expressing MC38 tumor cell via portal vein injection. On day 7 following tumor implantation, after an initial BLI measurement, mice were randomly divided into 6 groups and treated. Tumor growth was measured by BLI weekly and presented as the intensity of the luciferase signal. A, BLI signals represent tumor development in individual animals ($n = 5$ per group) before (week 1, first row) and after treatment (week 2, 3, and 4). Results shown are representative of 3 experiments. B, regions of interest were manually selected and quantification is reported as the average of photon flux within regions of interest. Each curve represents tumor development in a single mouse. Results shown are representative of 3 experiments. C, body weights were recorded every other day before and during treatment. The gains of body weight were compared with the body weight on the day before treatment. Each curve represents gain of body weight in individual mice. Administration of chloroquine (CQ) prevented the gain of body weight in IL-2-treated mice ($P < 0.05$). D, murine survival curve. When compared with the normal saline (untreated control, UT) group, HDIL-2 alone markedly prolonged survival time ($P < 0.01$). Chloroquine significantly enhanced HDIL-2 antitumor effects ($P = 0.024$).

other day during treatment. Although handling of mice receiving saline control typically is associated with initial weight loss, administration of HDIL-2 increased body weight, and this effect was prevented by combination with chloroquine ($p < 0.05$; Fig. 1C). Serum chemistry analysis suggested that 600,000IU IL-2 twice a day did not result in significant liver or kidney dysfunction (Supplementary Table S1), unlike that observed in patients.

HDIL-2 administration induces inflammatory cytokine release, which is inhibited by chloroquine

Our previous findings indicated that HMGB1 plays an important role in regulation of autophagy and apoptosis in

tumor cells. Translocation from the nucleus into the cytosol is associated with release of HMGB1 into the serum (33). Systemic release of HMGB1 may be related to the induction of systemic autophagy within tissues or cells, which are in turn associated with the toxicity of HDIL-2. We assessed HMGB1 distribution *in situ* in the liver as well as its serum level. Two hours following the last dose of HDIL-2 treatment (day 12 following tumor inoculation), liver tissues and serum were harvested. HMGB1 distribution, *in vivo*, and liver tissue immunofluorescent staining showed that HMGB1 was present predominantly in the nuclei of hepatocytes in untreated animals, as we have previously reported (31), but primarily located in the cytosol of hepatocytes in HDIL-2-treated animals with an

Table 1. Combination chloroquine with HDIL-2 prolongs survival time

Group	n	Survival time, d	Median survival time
PBS	10	27 × 3, 30, 31, 37, 38 × 2, 40, 55	31
Chloroquine	9	27, 31, 33, 34, 38 × 2, 42 × 2, 70	38
rIL-2 60K IU	5	31, 33, 38, 66, >150	38
rIL-2 60K IU + CQ	5	32, 38, 43, 68, 114	43 ^a
rIL-2 600K IU	9	50, 53, 55, 107, 135, >150 × 4	135 ^b
rIL-2 600K IU + CQ	10	66, >150 × 9	150 ^{b,c}

Abbreviation: CQ, chloroquine.

Compare with PBS control group: ^a $P < 0.05$, ^b $P < 0.001$.Compare with rIL-2 600K IU alone: ^c $P = 0.02$.

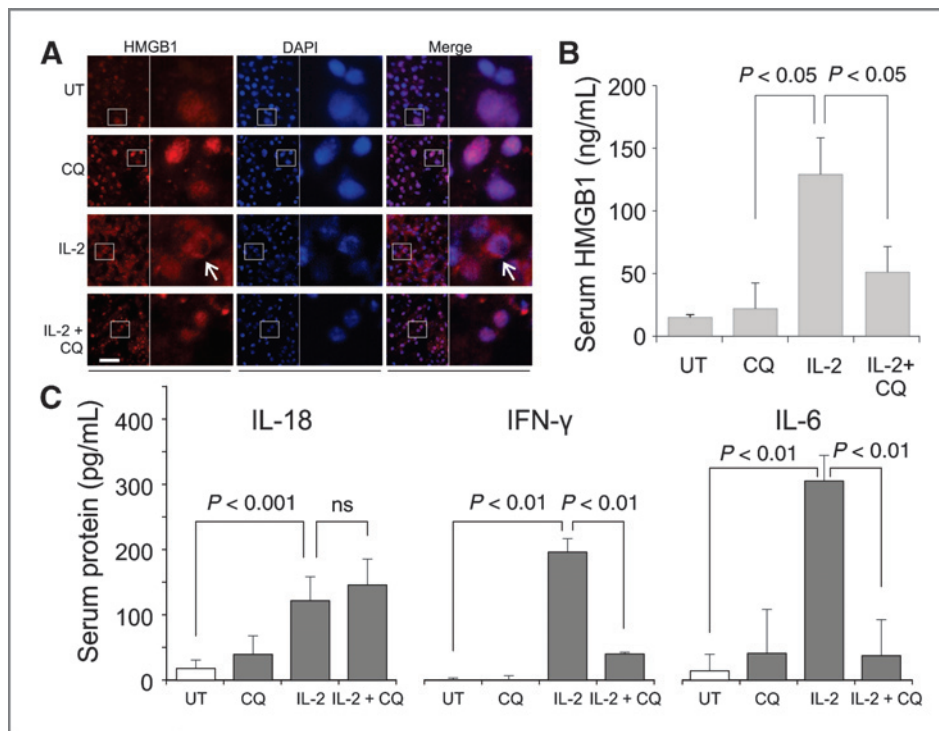
HMGB1 staining void within nuclei. Combination of chloroquine prevented HMGB1 translocation (Fig. 2A). This result is consistent with the change of HMGB1 serum levels in mice under different treatments. When compared with sham animals, tumor injection temporarily increases serum HMGB1 levels within 24 hours following tumor intraportal delivery, which then returns to normal levels (31). When compared with the UT control group, serum HMGB1 levels were significantly increased in animals receiving HDIL-2 treatment ($p < 0.05$; Fig. 2B). Conversely, combinations of chloroquine with IL-2 significantly decreased HMGB1 serum levels when compared with HDIL-2 alone ($p < 0.05$). These results suggest that chloroquine may limit HDIL-2-induced HMGB1 release thereby decreasing the associated systemic toxicity. Chloroquine administration alone did not change the serum levels of HMGB1.

Similar results were observed with other inflammatory cytokines. Compared with untreated control, HDIL-2 significantly increased levels of IL-6, IL-18, and IFN- γ in the serum. Administration of chloroquine inhibited levels of all cytokines except IL-18, which increased slightly. Thus the "cytokine storm" associated with the systemic toxicity of HDIL-2 were at least partially inhibited by chloroquine administration.

HDIL-2 significantly enhances immune cell proliferation and infiltration within the liver and spleen

Two hours following the last dose of HDIL-2 injection twice daily for 5 days, liver nonparenchymal cells and splenocytes were isolated, counted, and analyzed by flow cytometry. Consistent with our previous study, intrahepatic leukocyte numbers and splenocyte numbers were not changed following

Figure 2. Chloroquine (CQ) decreases HMGB1 release that is stimulated by administration of IL-2. On day 12 following tumor cell inoculation, 2 hours after the last dose of IL-2 injection, 3 mice from each group were sacrificed and livers and serum were harvested. A, immunofluorescent staining was carried out using rat anti-human HMGB1 antibody (red) and 4', 6-diamidino-2-phenylindole (DAPI) nuclear staining (blue). HDIL-2 induced HMGB1 translocation from the nucleus into the cytoplasm in hepatocytes (arrow). HMGB1 was restricted in the nucleus with combination of chloroquine. Image represents 2 independent experiments using samples isolated from 4 individual mice. Serum HMGB1 levels (B) and IL-6, IL-18, and IFN- γ (C) were measured by ELISA and diminished after chloroquine administration (except IL-18). Results shown are representative of 2 independent experiments.



intraportal infusion of MC38 tumor cells (31) but significantly increased by administration of HDIL-2. Flow cytometric analysis suggested that HDIL-2 significantly increased CD11c⁺, CD4⁺, CD8⁺, and CD11b⁺ cells (Fig. 3A, $p < 0.004$). Combination of IL-2 and chloroquine further enhanced this effect. There was also a modest elevation of Gr-1⁺/CD11b⁺ cells and CD4⁺/CD25⁺ cells as well as CD8⁺ cells ($p < 0.05$, Fig. 3B).

HDIL-2 administration induces profound mitochondrial changes and heightened autophagy

To assess the systemic effects of HDIL-2, (1.5×10^6 IU IL-2 twice a day) was administered and liver and kidney tissues from individual groups of mice were harvested and processed for TEM. When compared with UT animals, hepatocyte mitochondria from IL-2-treated mice had disrupted outer mitochondrial membranes and absent or disordered mitochondrial cristae. This could be a consequence of membrane provision to rapidly generate autophagosomes as has been reported in the setting of starvation (34) or a reaction to toxic reactive oxygen species generated during IL-2-induced stress. Increased endoplasmic reticulum (ER) surrounding the altered mitochondria were also visualized. The ultrastructure of the kidney proximal and distal tubule cells from both control and treated mice appeared normal (Fig. 4A). Furthermore, liver and kidney

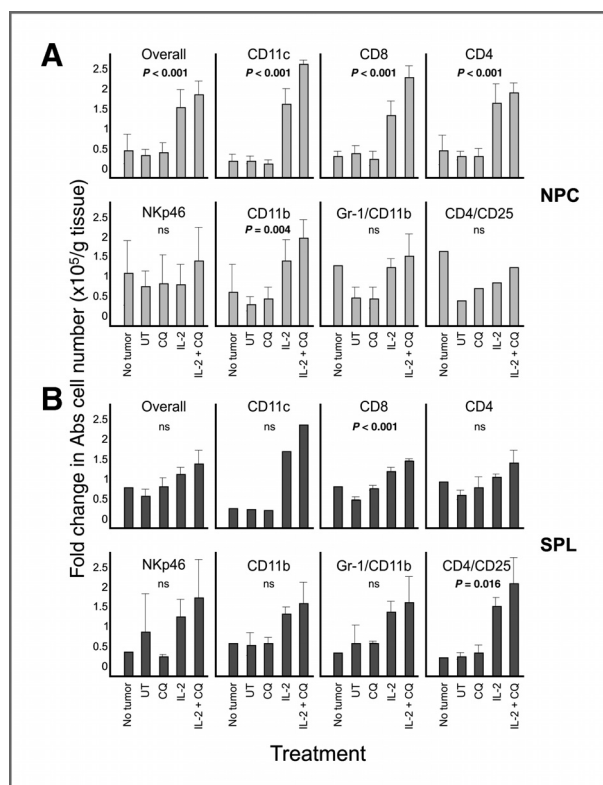


Figure 3. Administration of HDIL-2 and chloroquine (CQ)-stimulated immune cells proliferate and infiltrate into liver and spleen. Mononuclear cells from livers (A) and spleens (3 mice per group; B) were isolated. Cell numbers were counted and normalized by tissue weight. Cells were stained with individual antibodies and analyzed by flow cytometry. Data shown are representative of 1 of 3 similar experiments carried out. ANOVA P values are shown for each set.

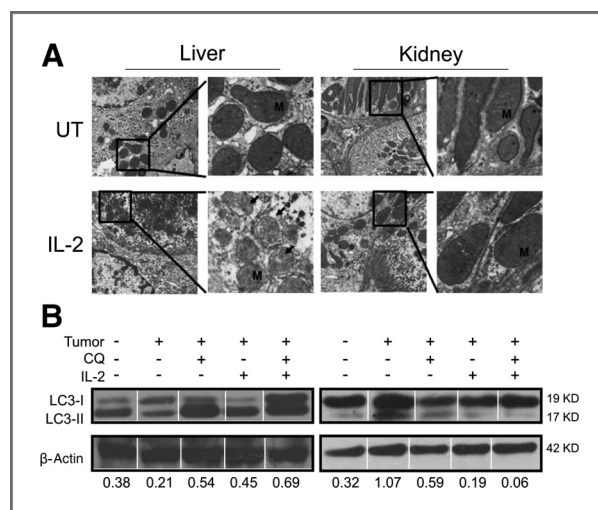


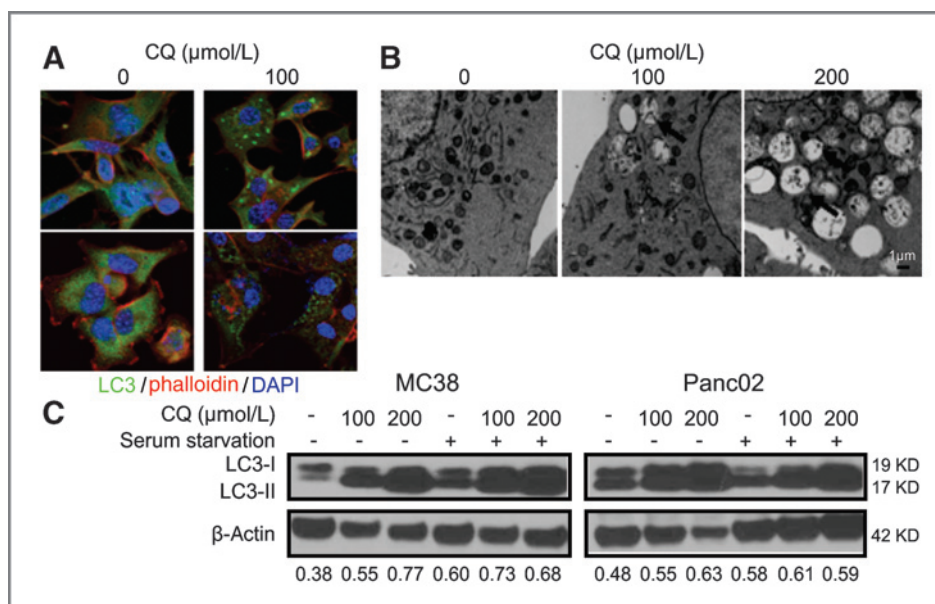
Figure 4. HDIL-2 administration promotes mitochondrial swelling and morphologic changes as well as autophagy *in vivo*. A, livers and kidneys were harvested from mice that received HDIL-2 (bottom row) treatment or UT (top row) as a control. Ultrastructure of liver and kidney was observed under TEM. Damaged mitochondrial that exhibited plasma membrane disruption; absent and disordered mitochondrial cristae (arrow) were observed in hepatocytes from IL-2-treated mice but not in control mice. The ultrastructure of a renal proximal tubule cell from both control and treated mice appeared normal. (M, mitochondria). B, Western blot analysis was conducted in liver and kidney tissue lysates collected from tumor-bearing mice that received IL-2 and/or in combination with chloroquine on the day after the last dose of IL-2 treatment (day 12). Enhanced autophagic flux was confirmed with increased LC3 II levels. Bottom numbers represent LC3-II/actin ratio. Data are representative of 1 of 3 experiments.

tissue lysates were collected from tumor-bearing mice that received IL-2 alone or in combination with chloroquine on the day following the last dose of IL-2. HDIL-2 treatment increased the apparent level of autophagic flux with enhanced conversion of LC3-I to LC3-II in the liver but not within the kidney tissue lysates, suggesting resistance of some tissues to the induction of autophagy. Chloroquine blocks the formation of autolysosomes, inhibiting the final steps of the autophagic process (35). Administration of chloroquine further enhances LC3-I/II levels in both groups of mice receiving chloroquine alone or in combination with IL-2. Enhancement of visualized autophagic vacuoles (termed autophagosomes) in treated animals were not observed (Fig. 4B).

Chloroquine inhibits autophagic flux in tumor cells

To determine whether chloroquine causes a similar effect on tumor cells, LC3 punctae were assessed in both MC38 and Panc02 cells. When compared with the untreated controls, chloroquine-treated tumor cells exhibited intense LC3 punctae by immunofluorescent staining (Fig. 5A). Under TEM, tumor cells appeared to have lost visible mitochondria and rough endoplasmic reticulum while accumulating autophagic vacuoles within the cytosol (Fig. 5B). To confirm these immune cytochemical observations, Western blottings were carried out on whole-cell lysates collected at 4 hours following treatment with either 100 or 200 μ mol/L chloroquine and the level of LC3-

Figure 5. Chloroquine (CQ) inhibits autophagy in tumor cells *in vitro*. MC38 and Panc02 tumor cells were cultured and treated with 100 or 200 $\mu\text{mol/L}$ chloroquine for 4 hours respectively. **A**, immunofluorescent staining was carried out for LC3 (green), actin (phalloidin, red), and nuclei (DAPI, blue). Chloroquine increased LC3 punctae in both MC38 and Panc02 cells. **B**, ultrastructure of tumor cells was observed under TEM. An accumulation of autophagosomes (arrow) were observed in chloroquine-treated cells. **C**, Western blot analysis for LC3-I/II showing a dose-dependent increase in MC38 tumor cells treated with chloroquine. Numbers shown below the Figure represent LC3-II/actin ratio. Data are representative of 3 experiments.



I and LC3-II assessed. A dose-dependent increase of LC3-II was observed in both MC38 and Panc02 cells (Fig. 5C). These results suggest that chloroquine induces accumulation of autophagic vacuole, inhibiting autophagic flux in both tumor cell lines.

Chloroquine treatment alters tumor cell metabolism

With TEM, in addition to accumulated autophagic vacuoles, we also found altered mitochondria that disrupted outer membrane and disordered mitochondrial cristae (arrow) in chloroquine-treated tumor cells (Supplementary Fig. S4). This suggested that chloroquine may alter tumor cell metabolism. Cells synthesize ATP solely through 2 pathways, mitochondrial respiration (oxidative phosphorylation, OXPHOS) and glycolysis. OXPHOS is more efficient, accounting for 90% of the ATP synthesized within normal cells. According to the Warburg hypothesis (36), tumor cells are surprisingly more dependent on glycolysis even in the presence of adequate oxygen and nutrients. In ATP production studies, blockade of glycolysis with 2-deoxy-D-glucose (2-DG) in MC38 and Panc02 tumor cells significantly diminished ATP levels but did not change when OXPHOS was blocked by oligomycin and rotenone (Supplementary Fig. S5). This has been attributed to both the need for substrate for anabolism and cell division (37) as well as the requirement of more rapid ATP generation by glycolysis in the setting of cell stress.

To show that chloroquine alters tumor cell metabolism, an XF bioenergetic assay system was used to explore the effects of chloroquine treatment on the bioenergetic phenotype of MC38 tumor cells by real-time monitoring of mitochondrial respiration in which OXPHOS is measured by oxygen consumption rate (OCR) whereas glycolysis is measured by the generation of lactate and the consequent extracellular acidification rate (ECAR). MC38 cells were exposed to 100 $\mu\text{mol/L}$ chloroquine for 4 hours, after which the effects of successive addition of oligomycin, FCCP, 2-DG, and rotenone, OXPHOS, and glycolysis rates were measured in real time. Chloroquine signifi-

cantly decreased baseline OCR in a dose-dependent fashion (Fig. 6A and B) but had no effect on baseline ECAR (data not shown). The addition of the complex V inhibitor, oligomycin, resulted in similar decrease in OCR with or without chloroquine addition. The mitochondrial uncoupler, FCCP, restored OXPHOS to levels above the baseline. 2-DG is a glucose analogue that inhibits hexokinase, the first enzyme in the glycolysis pathway, converting glucose to glucose-6-phosphate. Blockade of glycolysis by addition of 2-DG raised OCR slightly within control tumor cells because of OXPHOS compensation whereas the presence of chloroquine inhibited this effect. Addition of the complex I inhibitor rotenone significantly decreased both OCR and ECAR. These studies show that chloroquine decreases both OXPHOS and glycolysis within MC38 tumor cells, promoting a metabolic death. The agent ethyl pyruvate, which we have shown has antitumor activity and limits HMGB1 release, increased OXPHOS (Supplementary Fig. S6), suggesting that the OXPHOS decrease is not a generalizable phenomenon attributable to antitumor compounds.

As OXPHOS and glycolysis constitute the sole energy source of tumor cells, ATP levels were measured in the presence or absence of chloroquine. We found that ATP levels were markedly diminished following a short-term increase that may have resulted from compensatory increases in OXPHOS (Fig. 6C).

Chloroquine treatment induces tumor cell apoptosis

Chloroquine directly inhibits CT26 proliferation by inducing apoptosis both *in vitro* and *in vivo* (24). We showed that chloroquine inhibits MC38 and Panc02 cell proliferation and survival. The role of autophagy in tumor cell survival has not been elucidated completely, and the relationship between autophagy and apoptosis is complex, but in general, they are regulated reciprocally, with inhibitors of autophagy promoting apoptosis, perhaps by modulating mitochondrial pathways or mitophagy. Our findings indicate that chloroquine inhibits

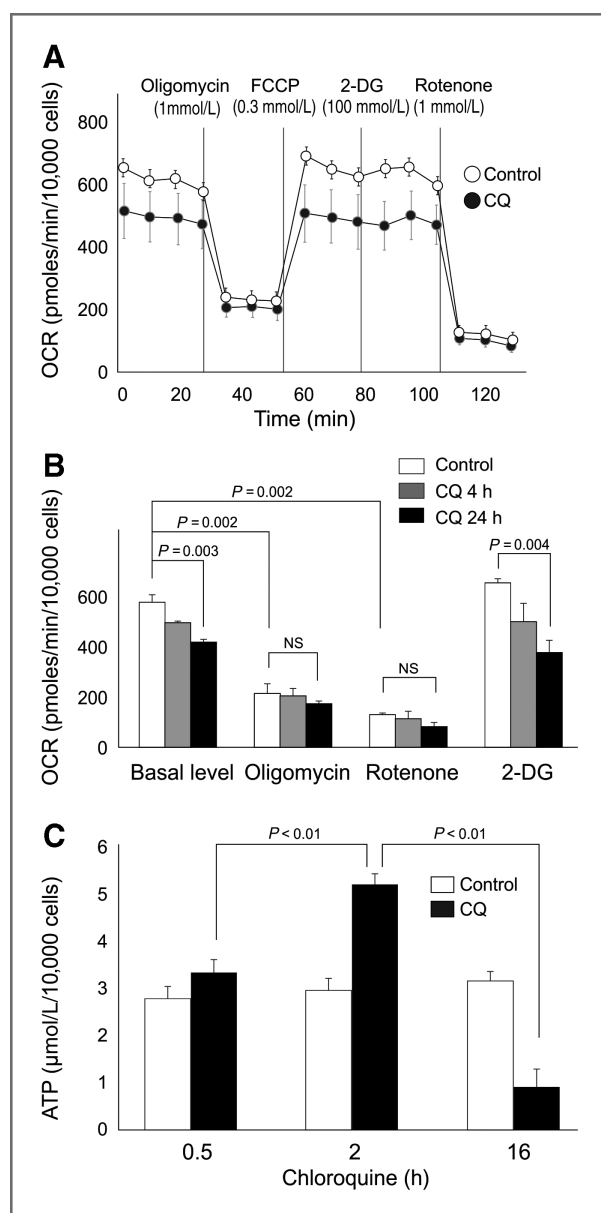


Figure 6. Chloroquine (CQ) alters tumor cell metabolism. A, OCR was measured in real time in a Seahorse XF bioenergetic assay. A total of 2×10^5 tumor cells were seeded with or without 100 $\mu\text{mol/L}$ chloroquine for 4 hours. B, average OCR was calculated from 3 measurements during the treatment of each compound (oligomycin, FCCP, 2-DG, rotenone) at the concentration as indicated. C, MC38 tumor cells were treated with 100 $\mu\text{mol/L}$ chloroquine for the time indicated. Levels of ATP production were plotted as mean with SDs from experimental replicates.

MC38 tumor cell autophagy. This effect may be related to induction of tumor cell apoptosis, given the complex interaction between the 2 processes. Addition of chloroquine to MC38 cells induced a significant dose-dependent increase in apoptotic cells, as showed by Annexin-V staining (Fig. 7A). Increased cleaved caspase-3 and cleaved PARP production were found by Western blotting (Fig. 7B). Enhanced release of cytochrome *c* into the cytosol in chloroquine-treated tumor

cells was also observed by both Western blotting and immunofluorescent staining (Fig. 7C and D). In aggregate, autophagy inhibition is indeed associated with induction of apoptosis in these cells.

Discussion

In the development of modern immunotherapy, experimentation in animal models has played an important role in advancing clinical trials in patients (38). Interestingly, the first murine tumor models used to show potent antitumor activity of IL-2 were methylcholanthrene-induced sarcomas with essentially no antitumor activity in humans with sarcomas. Similarly, although renal cancer and melanoma are the primary targets in humans, murine models show low activity of IL-2 alone in the Renca tumor model (although improved with the addition of IL-12 or IL-18) and with the nonimmunogenic murine melanoma, B16. It is perhaps best to consider, for many of these long-term cultured tumors, that the most important measure is of intrinsic immunogenicity rather than the tissue of origin. The liver is the primary site for metastasis in many epithelial tumors and, when unresectable, is associated with a high mortality rate. An hepatic model seems to more closely emulate metastases when compared with subcutaneous metastasis models (31). In this study, we established a reliable hepatic metastatic colorectal cancer model in mice using the intraportal route for injection. Using luciferase-labeled MC38 cells, enhanced tracking and better visualization of tumor growth is noted, allowing excellent correlation with data from pathologic examination and gross measurement of tumor bulk. This animal model permits sensitive detection and follow-up of hepatic metastases *in vivo*, allowing us to observe long-term antitumor effects of HDIL-2 immunotherapy and conduct mechanistic studies.

Although the precise mechanism(s) by which IL-2 mediates its anticancer effects is not fully understood, it is largely believed that it is affected by enhanced delivery to and activation of cytolytic effectors within tumor sites. Notwithstanding the presence of immune effectors, effective elimination of cancer often does not ensue, and the resistance has largely been attributed to effector dysfunction mediated by "exhaustion" (39) or the suppressive influences mediated by regulatory T cells (T_{reg} ; ref. 40) or myeloid-derived suppressor cells (41). We have shown that one of the major mechanisms of resistance resides in the target cells' enhanced autophagy and resistance to apoptosis (42). Here, we reported that administration of the autophagy inhibitor, chloroquine promotes substantial long-term antitumor effects when coupled with administration of IL-2. We also showed that IL-2 administration, in addition, causes not only release of HMGB1, a potent inducer of endogenous (33) and exogenous (43) autophagy, but also electron micrographic changes consistent with enhanced autophagy.

As a prototypic damage-associated molecular pattern (DAMP) molecule, HMGB1 plays a central role in the pathogenesis of many inflammatory states released following tissue damage or injury, and is found in the serum-including cancer (38–42) as well as other settings. In this model, we found that

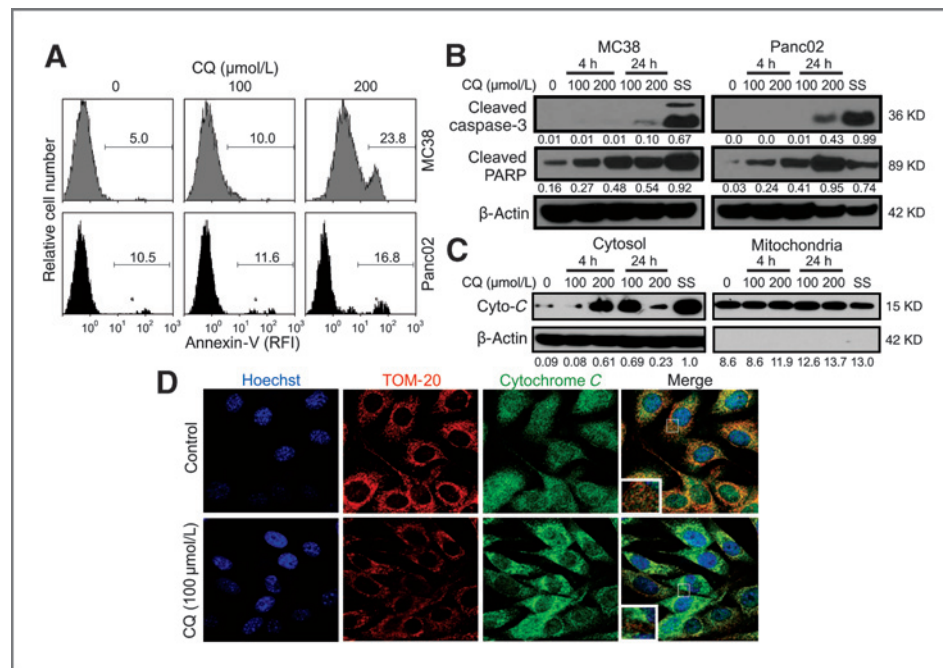


Figure 7. Chloroquine (CQ) induces tumor cell apoptosis associated with cytochrome *c* release from mitochondria. Tumor cells were cultured in the presence of 100 or 200 $\mu\text{mol/L}$ chloroquine for 4 or 24 hours before harvest. **A**, tumor cells exposed to 100 $\mu\text{mol/L}$ chloroquine for 4 hours and stained with Annexin-V/PI were analyzed by flow cytometry. **B**, Western blot analysis for cleaved PARP (C-PARP), cleaved caspase-3 showing a dose-dependent increase in apoptosis of MC38 and Panc02 tumor cells treated with chloroquine. Numbers presented below each figure represent cleaved caspase-3/actin or cleaved PARP/actin ratio. Results shown are representative of 3 experiments. **C**, protein from mitochondria and cytosol of chloroquine-treated MC38 cells were analyzed for cytochrome *c* (Cyto-*c*) by Western blot analysis, which showed the release of cytochrome *c* from mitochondria into cytosol. Cells untreated or under serum starvation were used as controls. Numbers presented below each represent Cyto-*c*/actin ratios. Results shown are representative of one of 3 experiments. **D**, MC38 cells were treated with 100 $\mu\text{mol/L}$ chloroquine for 24 hours then stained with cytochrome *c* (green), TOM-20 (mitochondrial marker, red), and Hoechst (blue). Chloroquine treatment increases cytochrome *c* release.

HMGB1 was released following HDIL-2 treatment and levels in the serum decreased following chloroquine treatment. We consider HMGB1 to be a likely candidate factor promoting the development of the systemic autophagic syndrome.

Chloroquine has been used for over half a century in humans for the treatment of rheumatoid arthritis, SLE, HIV and malaria. Chloroquine inhibits autophagy by blocking acidification of the lysosome, preventing fusion with the autophagosome (23). In a stressed cell, dependent on autophagy, this last step when blocked results in increased generation of autophagosomes, eventually undergoing either apoptotic or necrotic cell death. Recently, several studies have shown that chloroquine has extensive biologic effects, inhibiting cellular proliferation and/or inducing apoptosis in human and murine tumor cell lines such as the erythroleukemia K562 cells, breast cancer Bcap-37 cells (44), lung cancer A549 cells (27), ductal pancreatic adenocarcinoma cells, melanoma cells, C6 glioma cells (35), and mouse 4T1 cells. Autophagy inhibitors by themselves are unlikely to have significant clinical benefit, as only a small fraction of the tumor cells are under metabolic stress at a given time point. When combined with other chemotherapy agents, chloroquine enhanced cisplatin's cytotoxic effect and induced apoptosis by increasing the levels of intracellular misfolded proteins. In human cancer cell lines (24, 29) and murine models (30), chloroquine may exert significant antitumor activity by inhibiting the induction of

autophagy following cancer therapy. Chloroquine enhances chemotherapy and radiation sensitivity in clinical trials, the potential mechanisms underlying this enhancement are still unclear (45). Although Noman and colleagues (20) have recently shown that chloroquine administration promotes the effectiveness of antitumor vaccines in a murine model, ours is the first demonstration that it enhances the effectiveness of a cytokine therapy. Here, we also show that chloroquine limits autophagic vesicle degradation *in vitro* in two murine tumor cell lines. (Fig. 5).

We showed that chloroquine not only limited autophagy but also enhanced tumor cell apoptosis (Fig. 7). Induction of apoptosis was associated with the loss of mitochondrial membrane potential, release of cytochrome *c*, and activation of caspase-9 and caspase-3, and cleavage of PARP. *In vivo*, chloroquine significantly inhibited 4T1 tumor growth and metastasis in murine models and induced apoptosis in the tumor microenvironment (28). Autophagy also plays an important role in the effector-target interactions of cytotoxic cells and can confer a survival advantage for tumor cells targeted for cytotoxic cell killing. Autophagy may contribute to tumor resistance and perhaps to the toxicity observed during IL-2 cancer therapy (46). Our results suggest that inhibition of autophagy with chloroquine in combination with HDIL-2 treatment can increase the antitumor effects and increase survival when compared with IL-2 treatment alone.

Several studies have suggested that autophagy may act as a protective mechanism in tumor cells in which cell death is induced by drugs, and that inhibition of autophagy provides antitumor effects alone (22–24) or synergistic effect with such drugs (25). The role of metabolism in cancer is increasingly being appreciated (47). With the notion that conventional OXPHOS is suppressed, replenishing anapleurotic production of Krebs cycle substrates, enabling anabolism, and cell division is necessary in stressed cells. Enhanced autophagy promotes degradation of intracellular substrates and allows generation of amino acids, nucleotides, and lipids that can promote and enable subsequent replication. Here, we show that chloroquine (but not ethyl pyruvate) has the additional role of diminishing OXPHOS and limiting ATP production. This too may be part of the mechanism important in enhancing susceptibility in combination with IL-2 therapy.

Further work must evaluate the precise immunologic mechanisms operative in the IL-2 and chloroquine combination (Supplementary Fig. S7), defining which immune effectors (T cells or natural killer cells or both) have their activity promoted, whether autophagic inhibition limits immune reactivity through effects on dendritic cells, and define the precise role of HMGB1 in some of the biologic changes that we have shown. To that end, we recently created floxed HMGB1 mice and are testing its elimination in pancreas, dendritic cells, and natural killer cells. Recent studies (48, 49) suggest that autophagy is required for the immunogenic release of ATP from dying tumor cells, and increased extracellular ATP release improves the efficacy of chemotherapy when autophagy is disabled. Further understanding of the complex biologic roles of metabolism, autophagy, and immune effectors will allow development of more effective and less toxic regimens for patients with cancer. We have initiated a clinical protocol to test the delivery of IL-2 with the chloroquine congener hydroxychloroquine

in patients with advanced renal cell cancer based on these findings and our recent observations that IL-2 activated lymphocytes can themselves promote autophagy (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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